

# Glucose Transporter 1 Expression in Corneal Wound Repair under High Serum Glucose Level

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**Purpose:** To determine glucose transporter (GLUT) 1 mRNA and protein expression during corneal epithelial wound healing in diabetic rat.

**Methods:** Diabetes mellitus was induced by intraperitoneal injection of streptozotocin. At 10 days after injection, unilateral 3-mm epithelial debridement was carried out in the central cornea. At 2, 4, 6, and 24 hours after wounding, whole corneal epithelium was collected and GLUT1 protein and mRNA levels were determined by Western blotting and reverse transcription-polymerase chain reaction, respectively. Sugar content in collected samples was measured by the Anthrone reaction. Normal rats were used as controls.

**Results:** Glucose transporter 1 protein and mRNA levels in unwounded cornea were similarly low in the diabetic and control groups. Healing of corneal wounds was slower in diabetic rats than in controls. After wounding, GLUT1 mRNA and protein expression in both groups were similarly enhanced compared to unwounded epithelium. Sugar content at all time points did not show significant alteration in any group, although in diabetic rats it was significantly higher than in controls throughout the time course.

**Conclusion:** Glucose transporter 1 expression in diabetic rat cornea showed little difference from that in normal rat cornea, suggesting minimal influence of GLUT1 on the delayed healing of diabetic corneal wounds. **Jpn J Ophthalmol 2000;44:470-474** © 2000 Japanese Ophthalmological Society

**Key Words:** Cornea, diabetes, glucose transporter, wound repair.

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## Introduction

Corneal wound healing comprises several steps including migration of the epithelium to close the wound, cell proliferation allowing repopulation of the wounded area, and remodeling of the structure. These events are highly metabolic and depend on glucose as the main energy source. In the cornea, glucose is supplied from aqueous humor or glycogen stores in the epithelium.<sup>1</sup> It has been shown, however, that epithelial glycogen stores are dissipated rapidly in the migrating epithelium after debridement,<sup>2</sup> suggesting that an alternate source of glucose is present. In agreement with this, we have reported

that expression of glucose transport protein, glucose transporter (GLUT), was enhanced during wound healing in normal rat cornea indicating increased glucose supply to the epithelium.<sup>3</sup>

Glucose transporter is a facilitative transport protein that transports glucose into virtually all mammalian cells. To date, seven isoforms have been identified and named based on the order of cloning (GLUT1-7).<sup>4</sup> These isoforms vary in their tissue specificity, affinity for glucose, and whether their expression is inducible by insulin.<sup>4</sup> Among these isoforms, GLUT1 (50-55 kDa) is the most abundant and is expressed at high levels in erythrocytes and in brain tissue,<sup>5</sup> and is also considered to be the principal isoform in ocular tissue.<sup>6-12</sup> Glucose transporter 1 plays a vital role in cell survival and it is well-established that its expression is enhanced when cells are starved for glucose.<sup>13</sup> Thus, enhanced GLUT1 in

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normal wound healing indicates its essential role in healing processes. On the other hand, GLUT has been vigorously studied concerning its role in the pathology of diabetes mellitus. Glucose transporter is proposed to be one of the key factors controlling blood sugar levels and several investigators have reported its reduced expression in diabetic animals.<sup>14-16</sup> In diabetic cornea, although slow healing of epithelial wounds is well-known, its mechanism is yet to be elucidated. No studies have been performed to investigate if GLUT plays a role in the delayed healing in diabetic cornea. We hypothesized that decreased expression of GLUT1 during wound healing would be followed by reduction of glucose transport and deterioration of healing processes. The purpose of this study is to determine GLUT1 expression in the diabetic cornea.

## Materials and Methods

### *Animals and Wound Models*

Adult Sprague-Dawley rats were used and all procedures conformed to the ARVO Statement for the Use of Animals in Research. Diabetes was induced with a single intraperitoneal streptozotocin (60 mg/kg) injection. The control rats were injected with an equal volume of citrate buffer. At 10 days after injection, all rats were anesthetized with sodium pentobarbital and topical administration of proparacaine hydrochloride; then, a central area was demarcated unilaterally with a 3-mm trephine and the epithelium within the area removed with a small scalpel, leaving an intact basement membrane.<sup>17</sup> Wounds were allowed to heal in vivo. The high blood glucose level was confirmed on the day of wounding ( $n = 10$ ). To confirm elevated glucose environment, aqueous humor was also collected using a syringe with 30-gauge needle ( $n = 10$ ).

### *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

At the time points of 2, 4, 6, and 24 hours after wounding, rats were sacrificed with an overdose of sodium pentobarbital, and whole corneal epithelium from limbus-to-limbus was removed with a small scalpel. The epithelium removed to make the 3-mm wound was used as 0-hour sample. Ten eyes from each group were used for each time point. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method,<sup>18</sup> using isogen (Nippon Gene, Tokyo). Reverse Transcription-Polymerase Chain Reaction was performed with the superscript one-step<sup>TM</sup> RT-PCR System

(Life Technologies, Grand Island, NY, USA). With the template RNA of 0.4  $\mu\text{mol/L}$ , cDNA synthesis was performed at 50°C for 30 minutes and pre-denaturation at 94°C for 2 minutes. Polymerase Chain Reaction amplification was done with the following conditions; denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute with the specific primers for GLUT1 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (sequences were previously reported<sup>3</sup>). The optimal cycles of amplification were determined from the kinetics of PCR in preliminary experiments.

### *Western Blotting*

At the time points of 4, 6, and 24 hours after wounding, corneal epithelium was collected as described. Epithelium of 8 eyes from each group were used for each time point. Samples were solubilized in 1% sodium dodecyl sulfate (SDS) buffer containing protease inhibitors, analyzed by 10% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose paper. The paper was reacted with 1:500 dilution of goat anti-GLUT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then with 1:1000 dilution of horseradish peroxidase labeled anti-goat IgG (Santa Cruz Biotechnology). Antibody binding was detected by chemical reaction using 3, 3'-diaminobenzidine (Nacalai Tesque, Kyoto). Band densities were compared by image analysis.

### *Sugar Content Determination*

The supernatant of SDS buffer prepared for Western Blotting was treated with  $\text{H}_2\text{SO}_4$ , and its sugar content was measured by the Anthrone reaction<sup>19</sup> using a spectrophotometer at 620 nm ( $n = 8$  in each group at each time point). Sugar content was determined as D-glucose content by using D-glucose as the measurement standard. To compare the tissue sugar concentration in different volume, sugar content was determined as mg/mg protein.

## Results

All experiments described above were performed three times in different groups of animals, and the representative results are shown.

### *Diabetic State and Wound Healing*

Serum and aqueous humor glucose concentrations in diabetic rats were significantly higher than those in control rats (Figure 1). At 24 hours after wound-

ing, 85% (46/54) of the epithelial wounds were closed in control rats, while only 69% (37/54) were closed in diabetic rats. Corneal healing in diabetic rats was significantly slower than in controls ( $P < 0.05$ ,  $\chi^2$ -test).

### RT-PCR

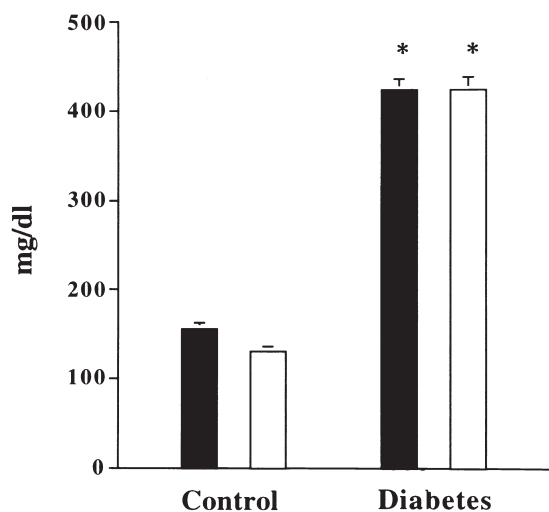
Based on the kinetics of PCR in preliminary experiments, 28 cycles of amplification were performed. Although the analysis is semi-quantitative, enhanced expression of GLUT1 mRNA was clearly detected at the 2-hour time point in each group (Figure 2). The expression remained enhanced at later time points. There was no apparent difference in GLUT1 mRNA levels between diabetic and control groups.

### Western Blotting

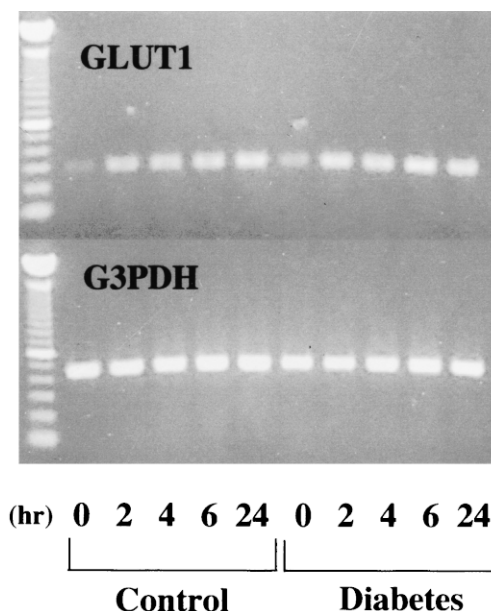
Glucose transporter 1 protein expression after wounding increased at the 4-hour time point coinciding with mRNA levels. No difference was shown between diabetic and control rats (Figure 3). Protein levels did not decrease until 24 hours after debridement.

### Sugar Content

Sugar content at all time points did not change markedly in either group, although it was significantly higher in diabetic rats than in controls throughout the time course (Figure 4).



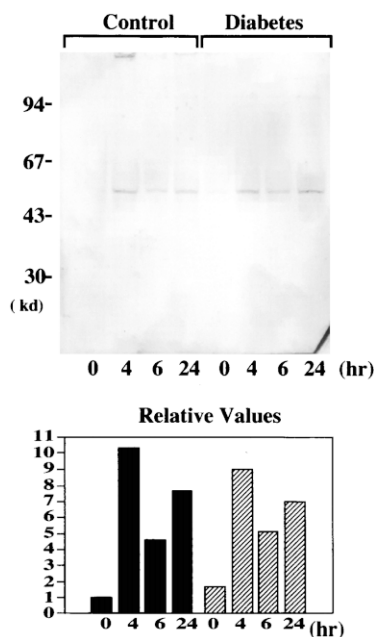
**Figure 1.** Glucose concentration of serum (■) and aqueous (□) ( $n = 10$ ). Glucose concentrations in diabetic group were significantly higher than in control. \* $P < .001$ , Student  $t$ -test, Bars = SD.



**Figure 2.** Reverse transcription-polymerase chain reaction. Twenty-eight cycles of polymerase chain reaction were performed. Glucose transporter 1 (GLUT1) mRNA expression increased rapidly after wounding and remained elevated at later time points. There was no marked difference between diabetic and control rats. Each lane contains samples from 10 eyes.

### Discussion

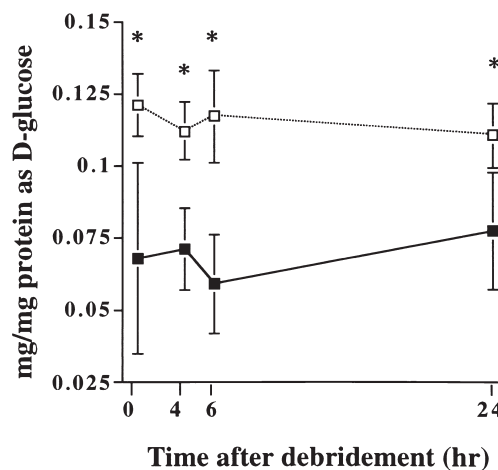
Diabetic corneal epitheliopathy is a clinical entity established rather recently. The phenomenon is most commonly observed clinically as poor epithelial healing after epithelial removal during vitrectomy. Although abnormal corneal innervation<sup>20,21</sup> or increased aldose reductase products in diabetic corneas<sup>22,23</sup> have been proposed as causes of slow healing, the mechanism is still poorly understood. Glucose transporter is considered to be one of the key factors controlling blood sugar level. Hypothetically, the most important GLUT isoform which is involved in the pathology of diabetes is GLUT4. GLUT 4 is mainly expressed in muscle and fat tissues and controls blood sugar level by changing intracellular glucose contents of those cells.<sup>24</sup> In fact, there have been several reports demonstrating downregulation of GLUT4 protein which was proposed to cause higher extracellular glucose level in diabetic animals.<sup>14-16</sup> In ocular tissue, however, it has been reported that GLUT4 expression was not detectable by Western blot or by immunohistochemistry.<sup>10</sup> In contrast, to GLUT4, which may induce a diabetic condition, GLUT1 expression in diabetes seems to be a cellular reaction to the elevated glu-



**Figure 3.** Western blotting. Glucose transporter 1 protein increased at 4-hour time point coinciding with mRNA expression. Protein levels remained elevated until 24-hour time point. No difference was found between two groups. Each lane contains samples from 8 eyes.

cose environment. To date, the organ most studied regarding GLUT1 in diabetes is the brain. In cerebral microvessels of diabetic rats, downregulated GLUT1 protein expression was reported,<sup>25-28</sup> and it is proposed that brain glucopenia in poorly controlled diabetic patients who are rapidly induced to normal glucose level is caused by reduced GLUT1.<sup>29</sup> Reduced protein with enhanced GLUT1 mRNA expression in diabetic rat brain was also reported.<sup>30</sup> Although this report suggested the posttranscriptional regulation, the mechanism of alteration of GLUT1 expression in diabetes is still unknown. In ocular tissues, an immunohistochemical study in diabetic human eyes<sup>11</sup> reported that corneal GLUT1 was normal. No quantitation, however, was performed and no study has been done concerning wound repair. As described previously, normal GLUT1 enhancement seems to be essential for smooth healing processes.<sup>3</sup> If GLUT1 expression is reduced or smooth enhancement during wound healing is hampered in diabetic cornea as in cerebral microvessels, glucose supply may be decreased and healing may be delayed. Thus, we sought to investigate GLUT1 expression in diabetic corneal wound healing.

In this study, we induced diabetes in rats by streptozotocin injection and performed experiments at 10



**Figure 4.** Tissue sugar levels. Although sugar content in diabetic group (□) was apparently higher than in control (■) at all time points. \* $P < .05$ , Student *t*-test, Bars = SD. Values did not change significantly throughout the time course in each group ( $n = 8$  at each time point in each group).

days after injection. Because diabetes is a chronic disease, there is concern that the diabetic state we induced may not reflect the chronic change caused by a true "diabetic" condition. It is more accurate to call the condition a high glucose state rather than a diabetic state. In previous studies, however, the altered expression of GLUT1 mRNA and protein in microvessels of rat brain was reported 7 days after streptozotocin injection.<sup>26,29,30</sup> As the results of these reports suggest the rapid adaptation of cells regarding GLUT1 expression to an elevated glucose condition, it seemed reasonable to employ a similar experimental diabetic model. Sugar levels in diabetic rats were significantly higher than in controls, agreeing with previous reports,<sup>31</sup> and the levels in either group did not apparently change throughout the time course. There was no apparent difference in GLUT1 mRNA and protein expression between diabetic and control rats in unwounded and healing cornea. Although the sugar content determined in this study reflects both intracellular and extracellular sugar content, the main contributing factor is thought to be the glycogen in the epithelium,<sup>31</sup> which is known to be dissipated in the healing epithelium after debridement under normal conditions.<sup>2</sup> It has been suggested that enhanced expression of GLUT1 compensates for the loss of glycogen stores.<sup>3</sup> Although how the intracellular sugar content changes after wounding in diabetes was not fully measured in this study, it can be safely assumed from the results that if the glycogen store is consumed as under nor-

mal conditions, glucose demand can be met by the enhancement of GLUT1. Recently, McDermott et al<sup>32</sup> reported that elevated extracellular glucose decreased migration of SV40-transformed human corneal epithelial cells. They speculated on the possibility of downregulated expression of GLUT1 and they also discussed factors other than nutrient availability as the cause of slow migration. Although there is a distinct difference between in vivo and in vitro conditions, we did not find decreased GLUT1, indicating that a mechanism other than simple energy supply plays a more important role in the slow healing of diabetic cornea. The diabetic model we made in this study was similar to those in previous reports in which reduced GLUT1 in cerebral microvessels was shown.<sup>25,26,28,29</sup> This suggests that our findings on normal GLUT1 in diabetic cornea do not reflect the model design but the property of the cornea itself. In conclusion, GLUT1 expression in diabetic cornea showed little difference from that in normal cornea, suggesting minimal influence of GLUT1 on the delayed healing of diabetic corneal wounds, at least in the early phase.

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